ISSN (print) 0093-4666 © 2015. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/130.629

Volume 130, pp. 629–639 *July–September 2015*

Russula sichuanensis and its ectomycorrhizae from Himalayan moist temperate forests of Pakistan

M. Saba* & A.N. Khalid

Department of Botany, University of the Punjab
Quaid-e-Azam Campus, Lahore, 54590, Pakistan

* Correspondence to: *rustflora@gmail.com*

Abstract — *Russula sichuanensis* and its ectomycorrhizae were collected from monoculture forests of *Pinus wallichiana*. This fungus is a new record for Pakistan and the ectomycorrhizae are described for the first time, based on morphology and nrDNA ITS sequences. *Russula sichuanensis* is a morphologically variable species especially with respect to the colour and shape of its pileus. The taxon is characterized by small to medium-sized stipitate basidiomata, reticulate globose to subglobose basidiospores, and pluriseptate pileocystidia. The ectomycorrhizae are distinguished by dichotomously branched orange-brown to grayish brown tips, its pseudoparenchymatous acystidiate mantle with irregularly shaped and often interlocking cells, and curved and frequently septate emanating hyphae. *Pinus wallichiana* is a new host for this species.

Key words — Khyber Pakhtoonkhaw, phylogeny, taxonomy, *Russulaceae*, *Russulales*

Introduction

The genus *Russula* Pers. is among the most diverse genera of *Agaricomycetes*. The genus includes approximately 750 species (Kirk et al. 2008), many of which are notorious for their intergrading colour variations and obscure morphological and anatomical discontinuities (Miller & Buyck 2002). Although thirty-three *Russula* species have been recorded from Pakistan (Ahmad et al. 1997, Niazi et al. 2006, Razaq 2013), ectomycorrhizae have been described only for *Russula brevipes* Peck (Niazi et al. 2006).

The aim of the present study is to elucidate the morphology and phylogeny of *Russula sichuanensis* and its ectomycorrhizae that form symbiotic associations with forest trees and play important role in the establishment and conservation of Himalayan forests.

During a mushroom survey in 2012 and 2013, seven different collections of morphologically different basidiomata and their ectomycorrhizae were made in Khyber Pakhtoonkhaw, Pakistan, which upon molecular analysis clustered together. Many of their morpho-anatomical features coincided with those of the Chinese species, *Russula sichuanensis*, and this identification was confirmed by ITS sequence analyses. *Russula sichuanensis*, a morphologically variable species, represents a new record for Pakistan, and its association with *Pinus wallichiana* has not previously been noted. Its ectomycorrhizae are described here for the first time.

Materials & methods

Morphological examination

Basidiomata were collected in 2012 and 2013, photographed, vouchered, dried, and characterized morphologically. All specimens are conserved in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH). Sections of specimens were mounted in 5% KOH for microscopic observations under a biological microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Tissues were mounted in phloxine to increase contrast of the structures, and Melzer's reagent was used to examine amyloid reactions.

Measurements of anatomical features (basidiospores, basidia, cystidia, pileus hyphae, and stipe hyphae) were made from at least 25 observations using an ocular micrometer and 100× oil-immersion objective; they include x = arithmetic mean of spore length and spore width for all spores measured, Q = spore length divided by spore width. Line drawings were made with a camera lucida. Color designations are from Munsell (1975).

DNA extraction, PCR amplification, DNA sequencing

Genomic DNAs were extracted from small pileus pieces using a modified CTAB method (Bruns 1995). Amplification of internal transcribed spacers (ITS1 and ITS2) and the 5.8S region of the nuclear ribosomal RNA gene were targeted using the primer pairs ITS1F/ITS4 (White et al. 1990; Gardes & Bruns. 1993) using the Extract-N-Amp plant DNA extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR was carried out under the following cycling parameters: initial denaturation (94°C for 1 min), 35 cycles (94°C for 1 min, 53°C for 1 min, and 72°C for 1 min), and final extension (72°C for 8 min). Amplified PCR products were sent for purification and bidirectional sequencing to Macrogen (Korea).

Sequence alignment & phylogenetic analysis

Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Following Dentinger et al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs 5′-(GAT) CATTA– and –GACCT (CAAA)-3′, and the alignment portions between them were included in analysis. All positions containing gaps and missing data were eliminated. There were a total of 406 positions in the final dataset. The analysis involved 37 nucleotide sequences. *Albatrellus ovinus* was used as outgroup based on results reported by Li et al. (2013).

Seven sequences from *Russula sichuanensis* basidiocarps, one sequence from an ectomycorrhizal root tip, and other related sequences retrieved from the GenBank were included in the phylogenetic analysis. The sequences were aligned by Muscle

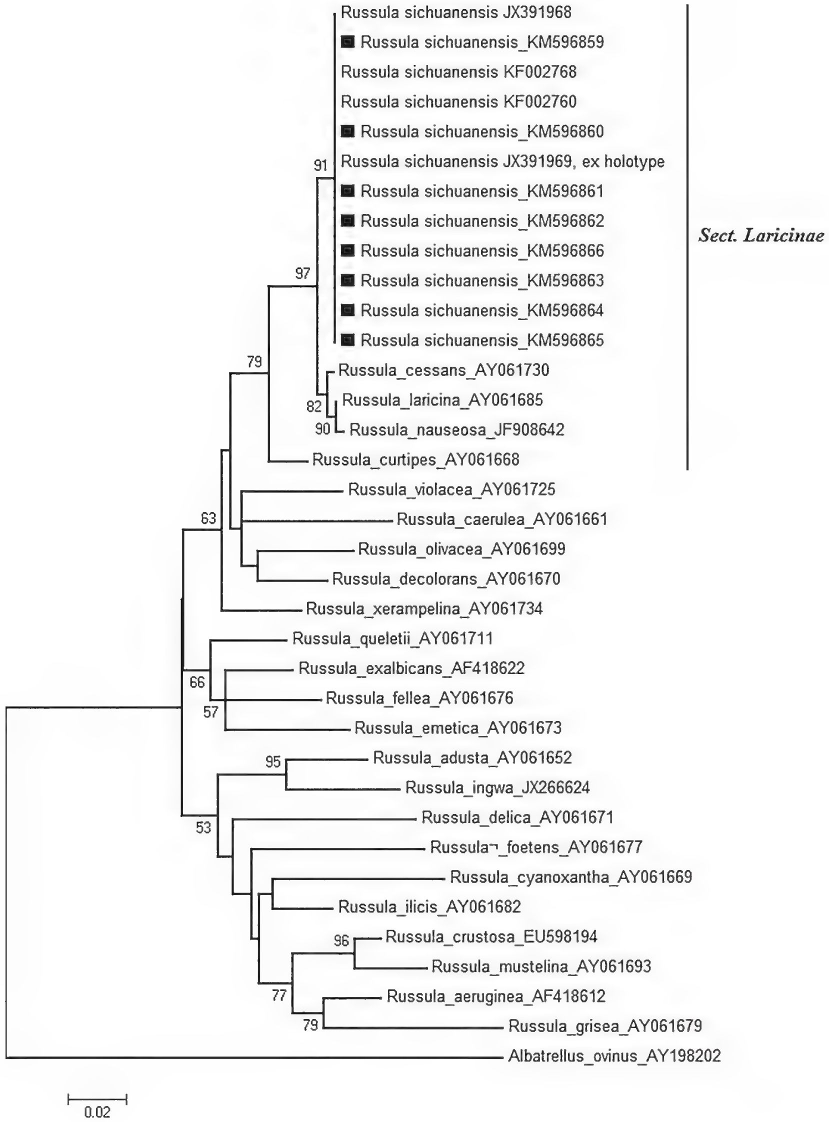
Russula sichuanensis JX391968
■ Russula sichuanensis_KM596859
Russula sichuanensis KF002768
Russula sichuanensis KF002760
■ Russula sichuanensis_KM596860
Russula sichuanensis JX391969, ex holotype
■ Russula sichuanensis_KM596861
■ Russula sichuanensis_KM596862
■ Russula sichuanensis_KM596866
■ Russula sichuanensis_KM596863
■ Russula sichuanensis_KM596864
■ Russula sichuanensis_KM596865
Russula_cessans_AY061730
Russula_laricina_AY061685
Russula_nauseosa_JF908642
Russula_curtipes_AY061668
Sect. Laricinae
Russula_violacea_AY061725
Russula_caerulea_AY061661
Russula_olivacea_AY061699
Russula_decolorans_AY061670
Russula_xerampelina_AY061734
Russula_queletii_AY061711
Russula_exalbicans_AF418622
Russula_fellea_AY061676
Russula_emetica_AY061673
Russula_adusta_AY061652
Russula_ingwa_JX266624
Russula_delica_AY061671
Russula¬_foetens_AY061677
Russula_cyanoxantha_AY061669
Russula_ilicis_AY061682
Russula_crustosa_EU598194
Russula_mustelina_AY061693
Russula_aeruginea_AF418612
Russula_grisea_AY061679
Albatrellus_ovinus_AY198202
91
97
79
82
90
63
66
57
95
53
96
77
79
0.02

FIG. 1. Phylogenetic relationship of *Russula sichuanensis* and closely related taxa based on Maximum Likelihood method inferred from nrITS sequences. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed.

(Beta) using the default setting in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2011). A phylogenetic tree was constructed with the Maximum Likelihood (ML) algorithm using a Jukes & Cantor (1969) model of nrITS sequences and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). The topology was assessed by 1000 bootstrap replicates. Corresponding bootstrap values >50% are cited on the tree figure. Percent Identities (PID) and DNA divergences were calculated by DNAStar.

The *Russula sichuanensis* sequences generated for this study were submitted to GenBank and the accession numbers for these and other closely related taxa used in the phylogenetic analysis are cited in phylogenetic tree (Fig. 1).

Results

Sequencing of the PCR products from the nrITS region of seven Pakistani specimens and one ectomycorrhizal root tip yielded fragments of 560–770 base pairs. Initial blast analysis of nucleotide sequences revealed a 99% maximum identity of the Pakistani sequences (GenBank KM596859–596866) with the sequence from the *Russula sichuanensis* holotype (GenBank JX391969). For phylogenetic analysis, several closely related taxa were selected from GenBank (Fig. 1).

After removing the ambiguous letters from the aligned datasheet, 1008 characters were subjected to phylogenetic analysis of which 294 characters were conserved, 428 were variable, 270 were parsimony informative, and 143 were singletons.

Percentage similarity was calculated by MegAlign (DNA Star Inc.). The Pakistani collections showed 98.6% identity and 0% genetic divergence with sequence JX391969 (*R. sichuanensis* holotype); 98% identity and 0% genetic divergence with sequence JX391968 (*R. sichuanensis* paratype), and 99% identity and 0.3% divergence with sequences KF002760 and KF002768.

Taxonomy

Russula sichuanensis G.J. Li & H.A. Wen, Mycotaxon 124: 179 (2013). Figs 2–4

Basidiomata small to medium sized, epigeous or semi-hypogeous, russuloid when mature. Pileus 15–62 mm diam., when young broadly parabolic, broadly convex with incurved margin and moderately to deeply indented centre or plane with flattened umbo, flesh grey upon drying, when mature straight or flaring to uplifted, tuberculate striate; surface dull, viscid, the centre very dark red (2.5R1/4) to very deep red (2.5R1/8), gradually less intense towards margin to dark red (2.5R3/4), the margin light grayish red (2.5R6/4) to deep red (2.5R3/10) or light olive brown (2.5Y5/2). Lamellae adnate or sinuate, regular, crowded, pale greenish yellow (7.5Y9/4) or light orange yellow (10YR9/8), margins even, concolorous. Lamellulae absent. Stipe 32–75 mm long, 7–11 µm wide above, 6–16 µm wide below, central, clavate or subcylindrical, tapering upwards, dry,

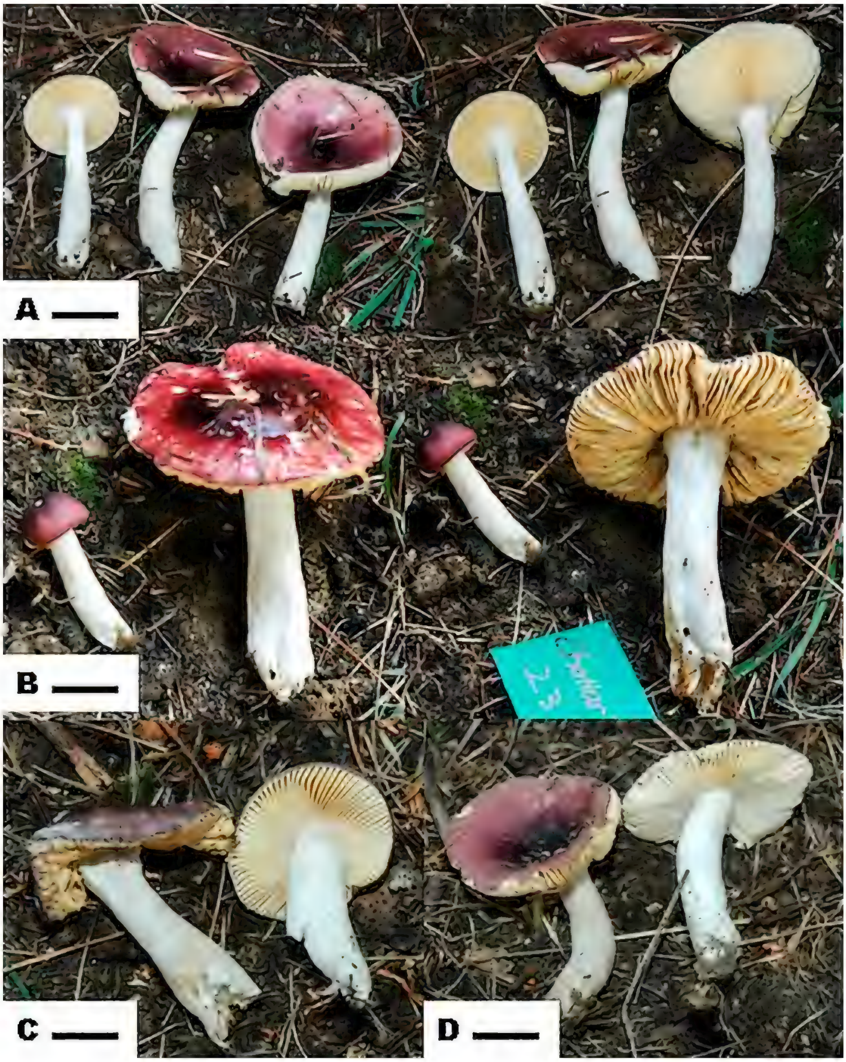

Fig. 2. *Russula sichuanensis* basidiomes: A, MSM0019; B, MSM0020; C, MSM0021; D, MSM0022. Scale bars-= 10 mm.

dull, longitudinally rugulose, stuffed at first, becoming hollow (cavernous) upon maturity, white with yellowish tinge. Annulus and volva absent. Odor and taste not recorded.

Basidiospores 8–12 × 7–10 µm [x = 9 × 7.7 µm, Q = 1.0–1.3], globose or subglobose, covered with amyloid warts linked as small crests and forming a

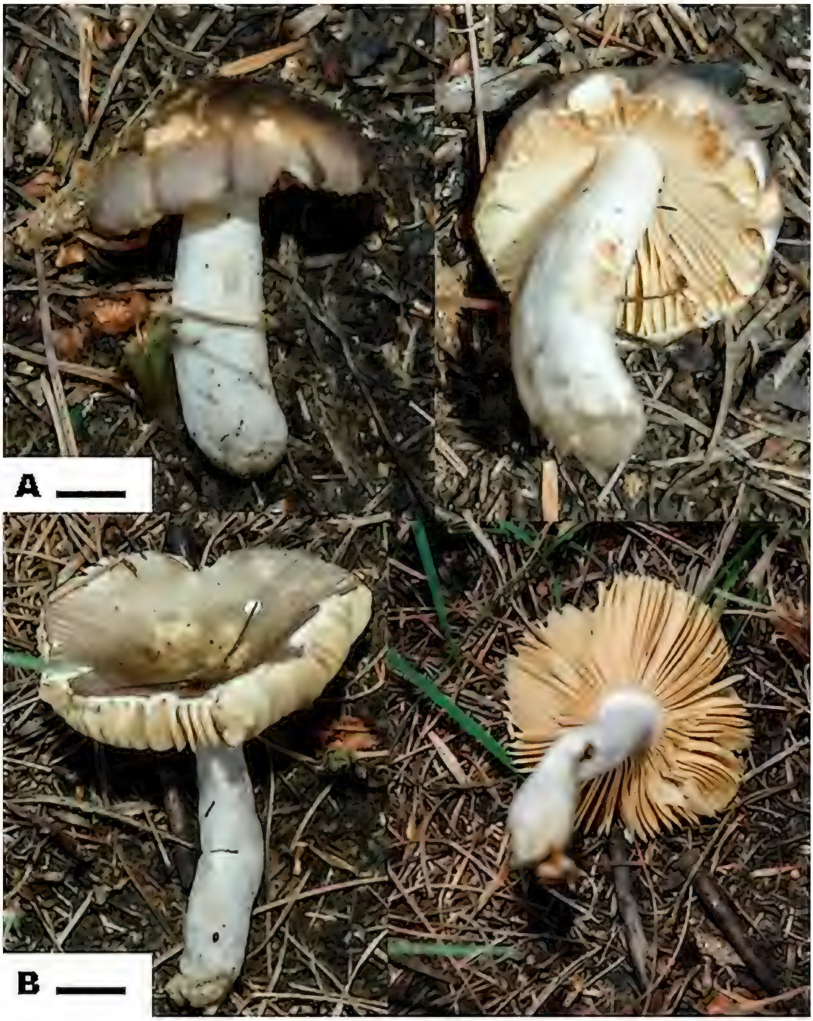

Fig. 3. *Russula sichuanensis* basidiomes: A, MSM0023; B, MSM0024. Scale bars = 10 mm.

partial network, rarely intermixed with isolated verrucae, warts often 0.5–1.5 µm high, thin-walled, yellowish in KOH, suprahilar plage well defined, 1.5–3.5 µm. Basidia 28–40 × 11–17 µm, clavate, bi- or tetra-spored, thin-walled, hyaline in KOH; sterigmata (2.5–)4.5–6 µm. trama cellular, cells inflated, thin walled, hyaline or yellowish in KOH 19–36 × 22–47 µm. Cheilocystidia absent. Pleurocystidia 42–78 × 8–16 µm, clavate or rarely mucronate with crystal

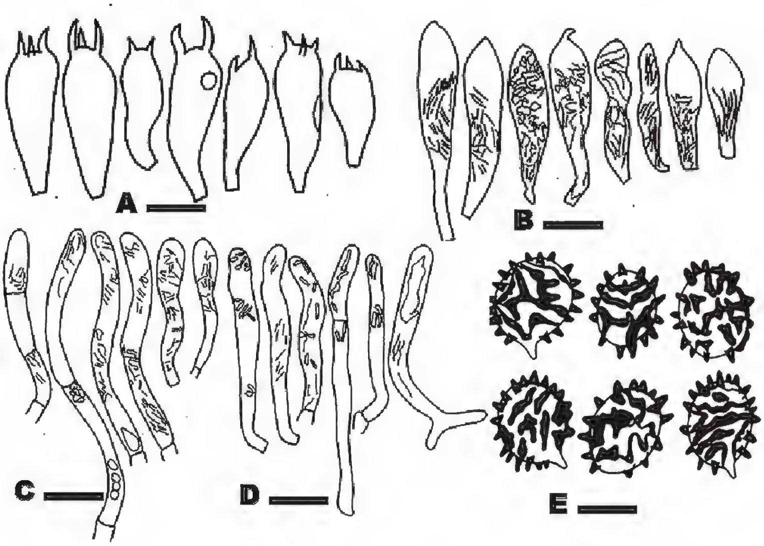

FIG. 4. *Russula sichuanensis* (MSM0019): A, Basidia; B, Pleurocystidia; C, Pileocystidia; D, Caulocystidia; E, Basidiospores. Scale bars: A = 20 µm; B–D = 25 µm; E = 8 µm.

or refringent contents, hyaline or yellowish in KOH, thin walled. PILEIPELLIS an ixotrichoderm composed of cylindrical hyphae, 3.5–6.3 µm diam., thin-walled, hyaline or greenish in KOH. PILEOCYSTIDIA with refractive contents, septate, clavate to cylindrical, 6.3–11.3 µm. STIPITIPELLIS interwoven, hyphae cylindrical, 3.3–5.3 µm, septate, hyaline in KOH. CAULOCYSTIDIA with refractive contents, septate, clavate to cylindrical, 56–89 × 6–10 µm. CLAMP CONNECTIONS absent.

MATERIAL EXAMINED (all in LAH): **PAKISTAN**, **KHYBER PAKHTOON KHAW**, Mansehra, Chattar plain, under *Pinus wallichiana* A.B. Jacks., 15 September 2012, M. Saba MSM0019 (GenBank KM596859), MSM0020 (GenBank KM596860); 22 September 2013, M. Saba, MSM0021 (GenBank KM596861), MSM0022 (GenBank KM596862), MSM0023 (GenBank KM596863), MSM0024 (GenBank KM596864), MSM0025 (GenBank KM596865); Shangla, ectomycorrhiza near rhizosphere of *Pinus wallichiana,* 2 September 2013, M. Saba MSE003 (GenBank KM596866).

Morphology of the ectomycorrhizae FIG. 5

ECTOMYCORRHIZAL SYSTEM: Dichotomous, ramified system 2.5 mm long. Unramified ends straight, 0.5 mm long, orange-brown to grayish brown and 0.5 mm in diameter (basal portion 0.4 mm wide; apical portion 0.5 mm wide). Very tips orange brown. Mantle surfaces smooth, matte, rarely giving rise to emanating hyphae and with host visible through mantle. Mycorrhizae orange-brown when young and grayish brown when old.

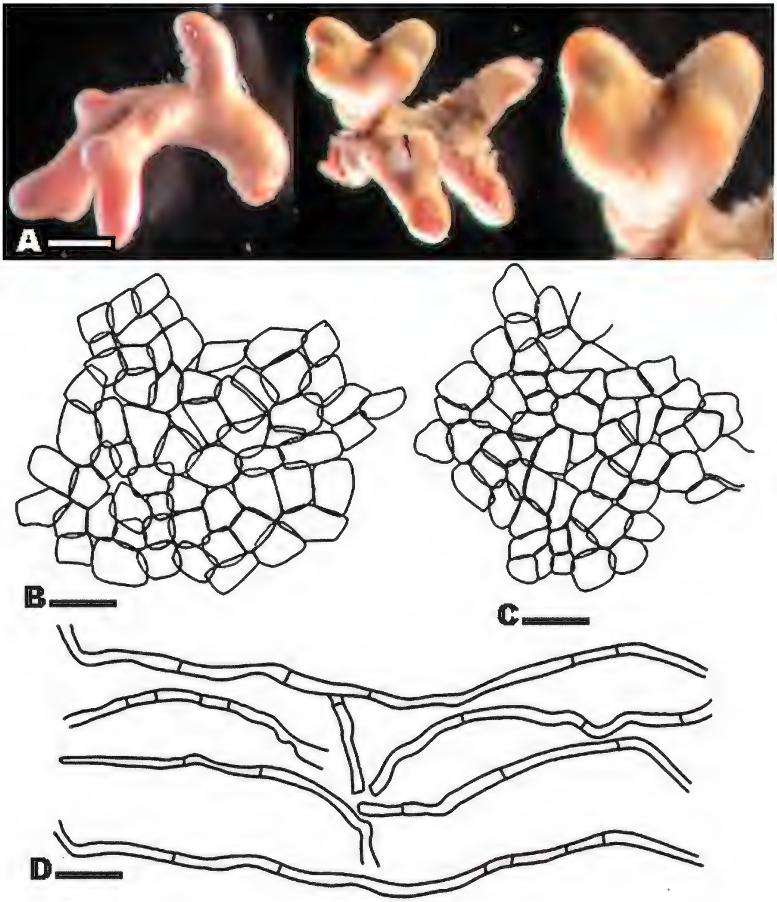

Fig. 5. *Russula sichuanensis* (MSE003) ectomycorrhizae: A, Morphotypes; B, Outer mantle; C, Inner mantle; D, Emanating hyphae. Scale bars: A = 5 mm; B–D = 15 μm.

Outer Mantle: pseudoparenchymatous (type L; Agerer 1987–2012) with irregularly shaped, often interlocking cells. Cells olive yellow to yellowish brown, 9.6–28.3 × 6.3–17.4 μm. Inner Mantle: pseudoparenchymatous (type L; Agerer 1987–2012) with irregularly shaped, often interlocking cells. Cells olive yellow to yellowish brown, 47–86.5 × 24–58 μm. Emanating Hyphae: curved, frequently septate, yellowish brown to olive yellow, thin walled, rarely branched, 8–25.9 × 2.4–4.1 μm. No clamp connection observed; H-type anastomosis observed among hyphae. Rhizomorphs: not observed. Cystidia: not observed.

Discussion

Russula sichuanensis was originally described by Li et al. (2013) from Sichuan province, China. It is characterized by an uneven pileus that often covers a white percurrent stipe and tightly crowded sinuate cream to yellow lamellae; it was additionally described as having a cream-tinged pileus (often olivaceous brown at the centre), a yellowish to orange sublamellate gleba, and stipitate basidiomata (Li et al. 2013). Our collections differ morphologically from the type description as follows: (1) curly and tightly contorted pilei were observed in only a few young basidiomata while the majority of mature basidiomata have broadly convex pilei with straight or uplifted margins and regular, straight (without convolutions) lamellae; (2) the pileus color is variable, ranging from a very dark red to very deep red centre, dark red gradually towards margin, light grayish red to deep red margin, or light olive brown. Most of the microscopic features, including shape, size of basidiospores, their ornamentation, presence of ixotrichoderm pileipellis with septate pileocystidia and septate caulocystidia, coincide with the description given by Li et al. (2013); however our collections had basidiospores with a well defined suprahilar plage and frequent pleurocystidia. Nonetheless, a BLAST comparison of all ITS sequences retrieved from our seven different basidiomata and one ectomycorrhizal root tip with the holotype of *R. sichuanensis* showed 99% identity and 100% query coverage and clustered with the holotype sequence and three other Chinese sequences in a clade with 91% support in phylogenetic reconstruction (Fig. 1).

Russula sichuanensis can now be characterized as a morphologically variable species, especially with respect to pileus colour and shape and which produces either a sublamellate gleba or regular lamellae. In addition, it can be characterized by partially small to medium sized stipitate basidiomata, subreticulate globose to subglobose basidiospores, and pluriseptate pileocystidia. The morphological variation in colour and forms result from non-genetic phenotypic plasticity and some edaphic or substrate factors may be involved in maintaining natural variants (Nicholl 1996; Petersen & Hughes 1999). Phenotypic plasticity in mushrooms has been largely unexplored, no doubt due to the ephemeral nature of most fungal basidiomata, which form only when environmental conditions are appropriate and then last only a short time.

Our phylogeny clusters *R. sichuanensis* with members of *Russula* sect. *Laricinae*, suggesting that it is closely related to the *R. laricina/cessans* group as previously depicted by Li et al. (2013). Typical morphological characters of this section include a cavernous stipe, tuberculate-striate cap margin, and flesh that does not yellow but greys after drying, all characters observed in our collections.

Russula mycorrhizae can be characterized by a pseudoparenchymatous outer mantle with either angular or irregularly shaped cells and which may be more or less densely covered with cystidia or acystidiate (Eberhardt 2002). In addition to the mantle features, Agerer (1999) defined a russuloid rhizomorph type, distinguished by its appression to a substrate (often to thicker roots) and differentiated into a peripheral layer with or without cystidia and central layer with vessel-like hyphae. According to Eberhardt (2002), ectomycorrhizae in *R.* subg. *Russula*, *Incrustatula*, *Tenellula* (in which *R. sichuanensis* falls), *Polychromidia*, and *Insidiosula* and in *R.* sect. *Felleinae* are acystidiate with a pseudoparenchymatous outer mantle that has irregularly shaped and often interlocking cells.

The ectomycorrhizal characters of *R. sichuanensis* are same as those of *Russula* subg. *Tenellula,* in which Li et al. (2013) described this species: dichotomously branched and orangish brown to grayish brown tips, a pseudoparenchymatous and acystidiate mantle with irregularly shaped and often interlocking cells and frequently septate, curved emanating hyphae. The presence of rhizomorphs was not noted.

Li et al. (2013) reported *R. sichuanensis* from conifer forests (*Picea* spp.); our study describes the basidiocarp and its ectomycorrhiza from monoculture *Pinus wallichiana* forests located in Himalayan moist temperate region.

Acknowledgments

We are highly indebted to Higher Education Commission (HEC), Pakistan, for funding this project under Phase II, Batch I, Indigenous PhD fellowships program for 5000 scholars. We are cordially grateful to Dr. Kanad Das (Cryptogamic Unit, Howrah, India), Dr. Sarah Bergemann, (Biology Department, Middle Tennessee State University, USA), Dr. Slavomír Adamčík, and Dr. Shaun Pennycook for critically reviewing the manuscript and giving their valuable comments. We are thankful to all lab fellows for accompanying us in the field trip.

Literature cited

Agerer R. 1999. Never change a functionally successful principle: the evolution of the *Boletales* s.l. (*Hymenomycetes, Basidiomycota*) as seen from belowground features. Sendtnera. 6: 5–91.

Agerer R (ed.). 1987–2012. Colour atlas of ectomycorrhizae, parts 1–15. Einhorn-Verlag, Schwäbisch Gmünd, Germany.

Ahmad S, Iqbal SH, Khalid AN. 1997. Fungi of Pakistan. Sultan Ahmad Mycological Society of Pakistan, Lahore.

Bruns TD. 1995. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant and Soil 170: 63–73. http://dx.doi.org/10.1007/BF02183055

Dentinger BTM, Didukh MY, Moncalvo JM. 2011. Comparing COI and ITS barcode markers for mushrooms and allies (*Agaricomycotina*). PLoS One 6(9): e25081. http://doi:10.1371/journal.pone.0025081

Eberhardt U. 2002. Molecular kinship analyses of the agaricoid *Russulaceae:* correspondence with mycorrhizal anatomy and sporocarp features in the genus *Russula*. Mycol. Prog. 1(2): 201–223.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113–118. http://dx.doi.org/10.1111/j.1365-294X.1993.tb00005.x

Jukes TH, Cantor CR. 1969. Evolution of protein molecules. 21–132, in: HN Munro (ed.). Mammalian Protein Metabolism, vol. 3. Academic Press, New York. http://dx.doi.org/10.1016/B978-1-4832-3211-9.50009-7

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Dictionary of the fungi. 10th Edition. CABI Europe–UK.

Li G, Zhao Q, Zhao D, Yue S, Li S, Wen H, Liu X. 2013. *Russula atroaeruginea* & *R. sichuanensis* spp. nov. from southwest China. Mycotaxon 124: 173–188. http://dx.doi.org/10.5248/124.173

Miller SL, Buyck B. 2002. Molecular phylogeny of the genus *Russula* in Europe with a comparison of modern infrageneric classifications. Mycological Research, 106: 259–276.

Munsell. 1975. Munsell™ soil color charts. Baltimore.

Niazi AR., Iqbal SH, Khalid AN. 2006. Biodiversity of mushrooms and ectomycorrhizas. 1. *Russula brevipes* Peck. and its ectomycorrhiza – a new record from Himalayan moist temperate forests of Pakistan. 38(4): 1271–1277.

Nicholl D. 1996. Relationships within the *Pleurotus djamor* species complex. Master's thesis. University of Tennessee, Knoxville, TN.

Petersen RH, Hughes KW. 1999. Species and speciation in mushrooms. Development of a species concept poses difficulties. Bioscience 49: 440–452.

Razaq A. 2013. Molecular characterization and identification of gilled fungi from Himalayan moist temperate forests of Pakistan using internal transcribed spacers (ITS) of rDNA. PhD thesis, Department of Botany, University of the Punjab, Lahore.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739. http://dx.doi.org/10.1093/molbev/msr121

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in MA Innis et al. (eds). PCR Protocols: a guide to methods and applications. Academic, New York.